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The Determination of Antithrombin III, α_2-Macroglobulin and α_2-Antiplasmin in Plasma by Laser Nephelometry[1])

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Summary: Specific assay procedures were developed to determine antithrombin III, α_2-macroglobulin, and α_2-antiplasmin in plasma with the aid of a Laser nephelometer. About 100 normal and pathological plasmas were examined and the results compared with those obtained by conventional immunological methods, such as radial immunodiffusion or rocket electrophoresis. Nephelometry proved to be a very rapid, precise, and reproducible method for the determination of antithrombin III and α_2-macroglobulin. The reproducibility of the α_2-antiplasmin assay was poor, probably as a consequence of the low concentration of this inhibitor in normal plasma.

Die Bestimmung von Antithrombin III, α_2-Makroglobulin und α_2-*Antiplasmin im Plasma mittels Lasernephelometrie*

Zusammenfassung: Zur Bestimmung von Antithrombin III, α_2-Makroglobulin und α_2-Antiplasmin im Plasma mittels Lasernephelometrie wurden spezielle Ansätze entwickelt. 100 Plasmen von gesunden und kranken Spendern wurden mit dem Lasernephelometer im Vergleich zu herkömmlichen immunologischen Methoden wie der radialen Immundiffusion und der Immunelektrophorese nach *Laurell* untersucht. Dabei erwies sich die Lasernephelometrie als eine schnelle, präzise und reproduzierbare Methode zur Bestimmung von Antithrombin III und α_2-Makroglobulin. Die Reproduzierbarkeit des α_2-Antiplasmin-Ansatzes war schlecht, wahrscheinlich infolge der relativ niedrigen Konzentration dieses Inhibitors im Plasma.

Introduction

Laser nephelometry has recently been presented as a very simple and rapid immunological method for determinations of proteins in solution (1). In order to test the applicability of a newly designed Laser nephelometric apparatus in a coagulation laboratory, we developed assays for the measurement of antithrombin III, α_2-macroglobulin, and α_2-antiplasmin. Plasma samples from healthy individuals and patients were examined, and the correlation with results obtained by conventional assay methods was investigated.

Materials and Methods

Apparatus

Laser nephelometer, manufactured by Behringwerke AG, Marburg, W.-Germany. For information concerning the principle of the apparatus, see l.c. (1) and l.c. (2).

[1]) Presented in part at the Fourth International Conference of Synthetic Fibrinolytic-Thrombolytic Agents, Carlsbad, Sep. 78

Plasma

Citrated blood was centrifuged at 1500 *g* for 10 min and plasma was stored at –20 °C not longer than 3 months. Plasma for standard curves was prepared by mixing plasmas from at least 10 healthy individuals. Pathological plasma samples were obtained from 48 patients suffering from liver disorders, acute venous thrombosis, disseminated intravascular coagulation, or acute leukemia, or from patients under fibrinolytic therapy.

Antisera

Antisera against antithrombin III and α_2-macroglobulin, specially prepared for Laser nephelometry, were provided by Behringwerke AG. The antiserum against α_2-antiplasmin was obtained through the courtesy of Dr. *D. Collen*, Dept. of Medical Research, University of Leuven, Belgium.

Radial immunodiffusion

Radial immunodiffusion of α_2-macroglobulin was performed according to the method of *Mancini* (3) on Partigen-Plates (Behringwerke AG).

Rocket immunoelectrophoresis

Rocket immunoelectrophoresis of antithrombin III was performed with a monospecific antiserum (Behringwerke AG) according to *Laurell* (4).

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Nephelometric assay

The principle of the nephelometric analysis is based upon *Heidelberger*'s fundamental experiments with antigen-antibody reactions in fluid media (5). In analogy to his findings, it was observed that the addition of plasma-dilutions with increasing concentrations of antigen to constant quantities of specific antiserum led to corresponding increases of measurable antigen-antibody complexes up to a certain maximum. This is known to be the point of antigen-antibody equivalence. When antigen in a relative excess reacts with its specific antibody, the amount of stable antigen-antibody complexes decreases compared with this maximum. As in nephelometry the concentration of an antigen is deduced from the quantity of antigen-antibody complexes, measured as suspended particles, an antibody excess is essential for the whole procedure. Generally, before establishing a standard curve, plasma dilutions with increasing concentrations are added to constant amounts of a specific antiserum to establish the adequate assay ranges.

Procedure

Both antisera and plasmas were filtered with disposable filters (Millex, 0,45 µm, Millipore, Neu-Isenburg, W. Germany) before use. Standard curves were prepared by diluting a pooled plasma with saline. After setting up the standard curve, the dilution of 1:16 was routinely used to examine a test plasma. The procedure employed for the estimation of antithrombin III and α_2-macroglobulin can be read from figure 1. Shortly, after filtering the plasma and antiserum dilutions, 0.1 ml plasma dilution was mixed with 0.2 ml antiserum dilution and incubated for 60 to 120 min in disposable LN-cuvettes (Behringwerke AG – W. Sarstedt GmbH, Nuembrecht/Rommelsdorf, W. Germany). Afterwards the nephelometric analysis was performed without use of a blank (2).

In the assay of antiplasmin, plasma and antiserum were employed in higher concentrations than usual because of the low concentration of α_2-antiplasmin in normal plasma. Antiserum was diluted 1:2 in saline. With this exception the assay corresponded to that of antithrombin III.

Results

Antithrombin III

With plasma dilutions from 1:8 to 1:64 a reproducible standard curve with an adequate assay range could be set up for antithrombin III determinations. As can be seen in figure 2, the values of this standard curve partly depended on the incubation period employed. With the antiserum used in these studies, higher and more reproducible signals, especially for the higher plasma dilutions, could be obtained after 2 hours instead of 1 hour incubation time. The coefficient of variation, calculated on the basis of 20 serial measurements with 120 min of incubation, was 4.6% in plasma dilutions 1:64 and 1.2 % in dilutions 1:16. The correlation of the nephelometric assay with rocket-electrophoresis was studied on parallel measurements of 48 pathological plasma samples. A high degree of correlation was demonstrated (fig. 3).

The normal range, defined as the mean value ± 2 SD and calculated on the basis of nephelometric determinations of antithrombin III in 50 plasmas of healthy adults, was located between 215 and 308 mg/l.

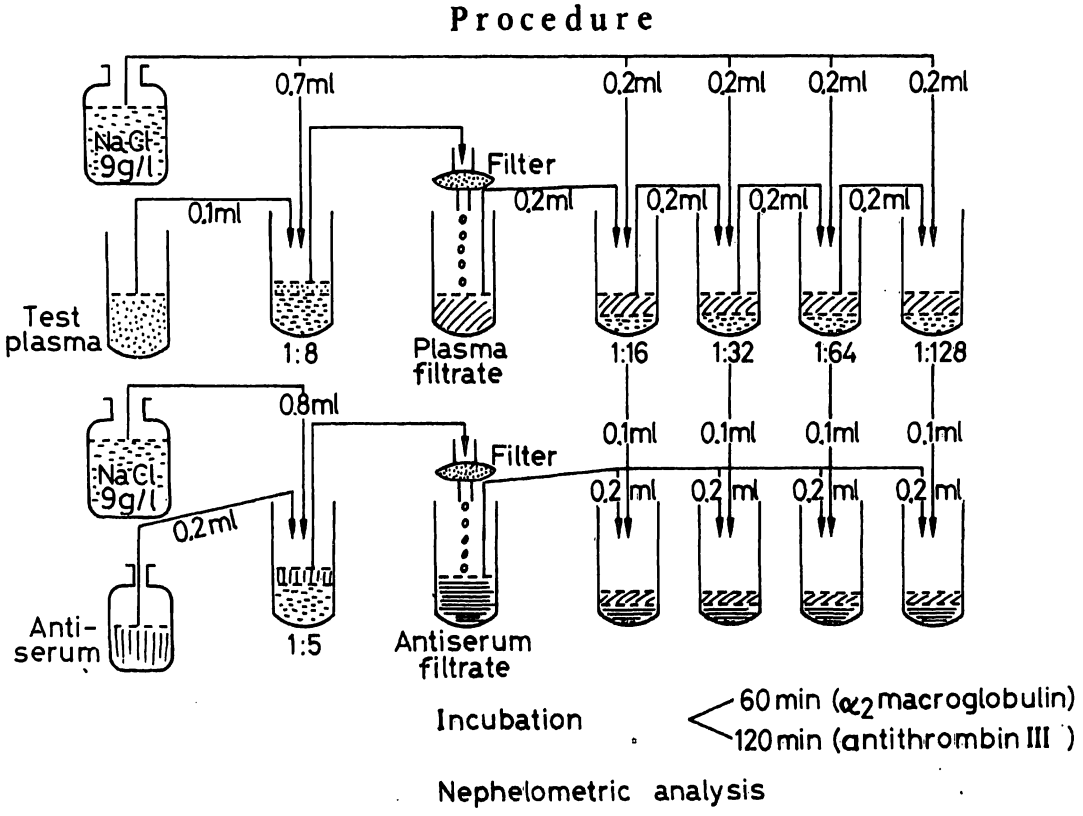

Fig. 1. Assay for the nephelometric analysis of antithrombin III and α_2-macroglobulin.

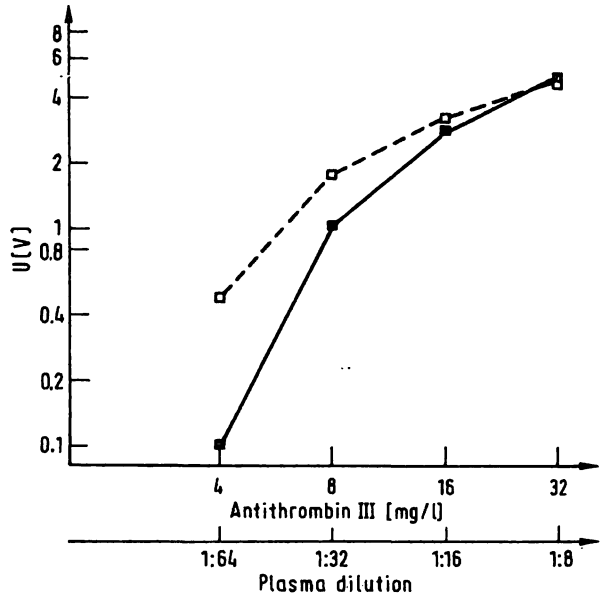

Fig. 2. Standard curve for antithrombin III determination.
Ordinate: Scattered light in volts(V).
Abscissa: Concentrations of antithrombin III in the dilutions of pooled normal plasma.
Incubation ■—■ 60 min, □—□ 120 min

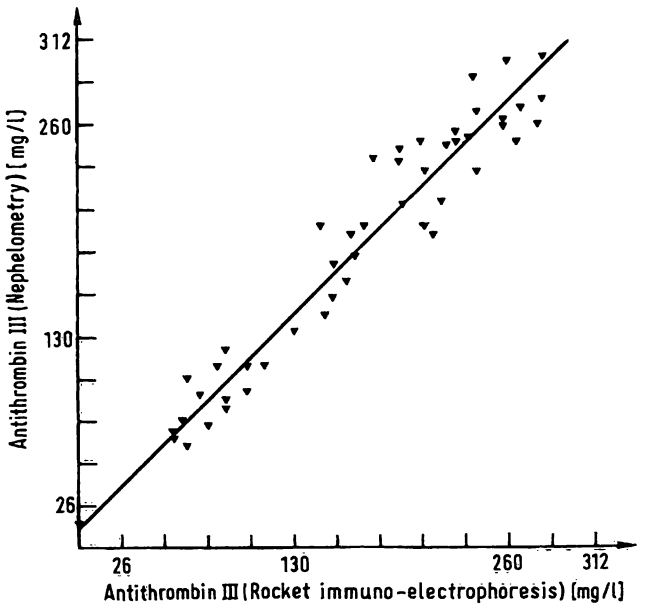

Fig. 3. Determination of antithrombin III in pathological plasmas. Correlation of Laser nephelometry with rocket electrophoresis (*Laurell*).
r = 0.965 y = 1.019 · x + 11.5 n = 48

The antithrombin III content of the undiluted standard plasma employed in these investigations was 259 mg/l.

α_2-Macroglobulin

A very reproducible standard curve could be established for this proteinase inhibitor (fig. 4). It is almost linear and covers an assay range similar to that of the antithrombin

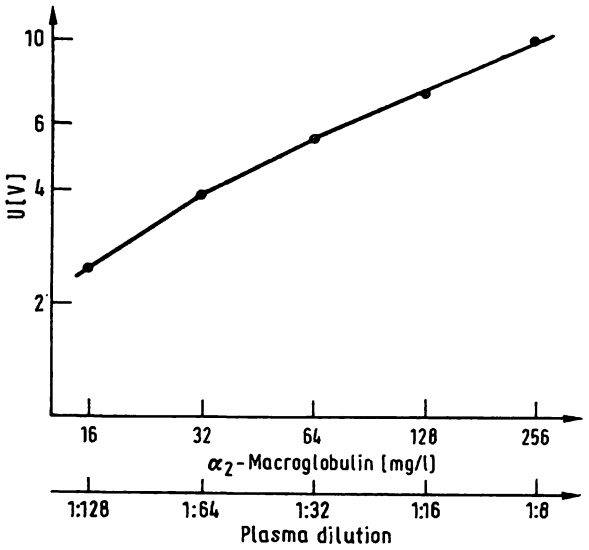

Fig. 4. Standard curve for α_2-macroglobulin determination.
Ordinate: Scattered light in volts (V).
Abscissa: Concentrations of α_2-macroglobulin in the dilutions of pooled normal plasma

III curve. The coefficient of variation was 4.9 % for plasma dilutions 1:128 and 3.9 % for dilutions 1:16.

Parallel determinations of the α_2-macroglobulin concentration in 48 pathological plasmas by laser nephelometry and radial immunodiffusion showed an excellent correlation (fig. 5). The normal range, calculated on the basis of nephelometric determinations of α_2-macroglobulin in 50 plasmas of healthy adults, was located between 1.14 and 2.95 g/l. The undiluted standard plasma employed in these investigations had an α_2-macroglobulin content of 2.04 g/l.

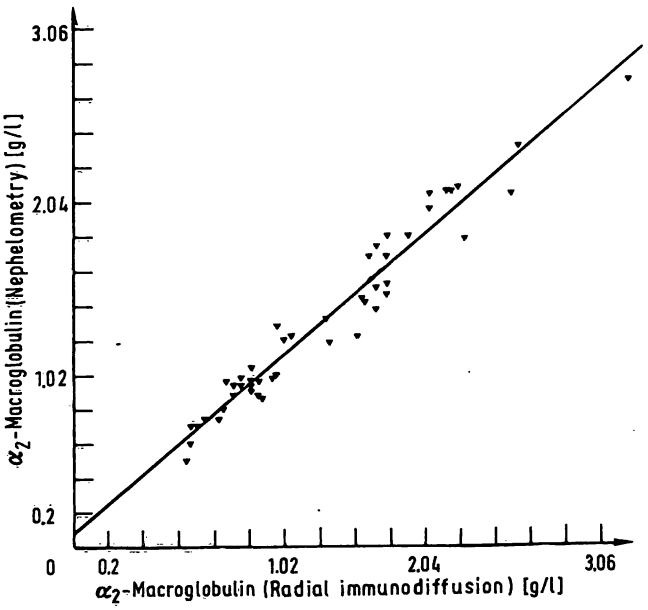

Fig. 5. Determination of α_2-macroglobulin in pathological plasmas. Correlation of Laser nephelometry with radial immunodiffusion.
r = 0.972 y = 0.879 · x + 0.076 n = 48

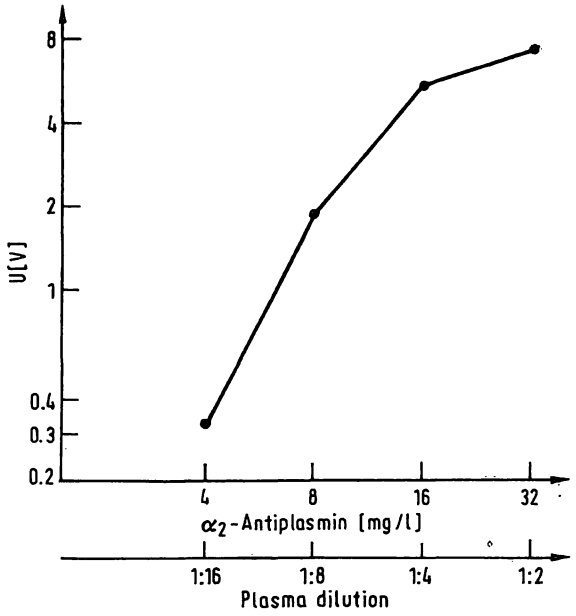

Fig. 6. Standard curve for α_2-antiplasmin determination.
Ordinate: Scattered light in volts (V).
Abscissa: Concentrations of α_2-antiplasmin in the dilutions of pooled normal plasma.

α_2-Antiplasmin

The establishment of an adequate standard curve for α_2-antiplasmin, similar to those of antithrombin III and α_2-macroglobulin, proved to be impossible because of the low concentration (60–70 mg/l) of this inhibitor in normal plasma (6,7). In order to augment the concentration of measurable antigen-antibody complexes in the assay, the antiserum was used in a dilution of 1:2 instead of 1:4, so that the test-plasma could also be employed in higher concentrations (fig. 6). By this procedure, normal contents of α_2-antiplasmin were easily measurable. However, in plasmas with lower α_2-antiplasmin concentrations, the minute amounts of antigen-antibody complexes in solution could not be detected with sufficient accuracy. The coefficient of variation reached 9% in serial determinations of the plasma dilution 1:4 and far over 10% when plasma was diluted 1:16. In view of the poor reproducibility of the assay we decided not to examine pathological plasmas.

Discussion

At present many plasma proteins are being routinely determined by immunological methods. Some of them, for example the proteinase inhibitors examined in this paper, can also be assayed functionally; this, in our opinion, should be regarded as complementary, rather than as an alternative. Laser nephelometry, a quantitative immunological method, has recently been introduced as a very simple and time-sparing way of estimating proteins in serum and plasma. In the course of the experiments, which we performed in normal and pathological plasmas, we were confronted with some of the advantages and limitations of this procedure.

For the determination of antithrombin III and α_2-macroglobulin, standard curves with good reproducibility could be established. The results obtained in serial α_2-antiplasmin determinations were less satisfactory, with coefficients of variation sometimes higher than 10 %. This is probably due to the restricted sensitivity of the method. α_2-Antiplasmin is known to be found in relatively low concentrations in normal plasma (6,7). In our experiments we could demonstrate that α_2-antiplasmin determinations with reasonable coefficients of variation could still be performed down to a concentration of about 15 mg/l plasma. When the concentration of this inhibitor was lower than 5 mg/l the coefficient of variation exceeded 10%. However, these results are not necessarily valid for other proteins; it can be assumed that the sensitivity of the method largely depends on individual properties of an antigen and the avidity of its antibody. Accordingly, the standard curves presented in this paper should only be regarded as examples. Their actual course varies with the quality of the antisera employed.

The results of the antithrombin III and α_2-macroglobulin determinations correlated excellently with those obtained by rocket electrophoresis and by radial immunodiffusion. Obviously the normal ranges found with Laser nephelometry correspond with those published by authors (8,9) who worked with different immunological assays.

With regard to the technique of the nephelometric assay, both the rapidity of the procedure, which normally takes about 2 hours, and its simplicity can be considered as decisive advantages in comparison with conventional immunologic methods.

As a conclusion, it may be stated that laser nephelometry is a simple and rapid immunologic procedure for the determination of proteins in plasma. The method is sensitive enough to detect pathological deviations with satisfactory precision, provided that the normal concentration of the protein to be analysed exceeds 100 mg/l plasma.

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